Antioxidant and Antimicrobial Activities of Naturally Occurring Flavonoids from *M. heterophylla* and the Safety Evaluation in Wistar Rats

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A B S T R A C T

Background:

Maytenus heterophylla (M. heterophylla) is commonly used in African traditional medicine for the management of various ailments. The present study evaluated the antioxidant, antimicrobial and safety properties of the Flavonoid extract of *M. heterophylla* in Wister rats.

Methods:

The Flavonoid was subjected to antibacterial study via agar well diffusion method, and antioxidant study using 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant properties (FRAP) assays. Subacute toxicity were carried out by the oral administration of the extract at a daily dose of 50 or 100mg/kg for 28 days.

Results:

The extract produced significant antioxidants activities with IC_{50} of 33.07±0.84 µg/mL & 38.08±0.89 µg/mL in DPPH and FRAPS models respectively. It produced a dose-dependent inhibition of *S. aureus, E.coli, P. aeruginosa, K. pneumonia* and *S. Typhi* with MIC between 12.5µg/mL to 25µg/mL. The flavonoid was safe on acute exposure to rats (LD_{50}> 5000 mg/kg). However, the chronic exposure significantly ($p<0.05$) decreased the creatinine, bilirubin concentrations and increased aspartate transaminase (AST) activities while the total protein, albumin, alanine transaminase (ALT), alkaline phosphatise (ALP), urea, chloride, potassium and sodium concentrations were comparable with those in the controls. The organs-body weights ratios also compared well with the controls ($p<0.05$).

Conclusions:

The findings showed that the Flavonoid extract of *M. heterophylla* was relatively non-toxic following acute or chronic exposures at 50-100 mg/kg. The flavonoid extract may potentially serve as a candidate agent for the development of an anti-microbial drug and to enhance the antioxidant capacity in rats.

Keywords:

Antibacterial; Anti-Oxidants; Flavonoids; Maytenus Heterophylla; Toxicity

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INTRODUCTION

Infectious diseases due to bacteria are serious health concerns worldwide (1). Of the annual death rate of 52 million, over 17 million of them are attributed to infectious diseases, including about 9 million deaths in young children (2). The rise in bacterial resistance to conventional antimicrobials is a major healthcare problem in the management of infectious diseases (3).

The implicative role of free radicals in many pathological conditions, such as liver disease, diabetes, inflammation, coronary heart diseases, carcinogenesis,

drug toxicity and neurodegenerative disorders like Alzheimer and Parkinson diseases has been well established (4,5). Redox reaction in bodily systems can lead to the generation of free radicals, which initiate chain reactions that cause cellular damages. However, antioxidants cease the reaction chain by removing the intermediates radicals and preventing further oxidation reactions (5). The development of resistance as well as side effects and toxicity associated with conventional antioxidant and antimicrobial drugs call for an alternative safe and cost-effective therapy (6).

Natural products particularly from medicinal plants have demonstrated their significance as sources of metabolites with therapeutic virtues, and are considered as reliable candidates for the discovery of novel phyto-pharmaceuticals with various biological activities, such as anti-parasitic, antimicrobial and antioxidant effects (5, 7-9). The high interest in natural antimicrobials and antioxidants in safeguarding the human body from oxidative stressors and infectious diseases is increasing around the globe due to their efficacy, less side effects and high safety as opposed to those for synthetics drugs (10). Thus, natural products are the main spotlight of researchers for the isolation of antimicrobial and antioxidant derivatives that can modulate metabolic pathways and promote health and well-being in humans (11).

Maytenus heterophylla (M. heterophylla) is an African shrubs commonly known as spike thorns, and is a medicinal plants commonly used by the traditional healers to treat respiratory disorders, snake bites, wounds and dysentery (12). Previous studies have established that plasmodial, leishmanial (13,14), inflammatory (15) and bacterial (16) conditions can be treated with *M. heterophylla*. Also, phytochemical studies have identified flavonoids, alkaloid, terpenoid and triterpenes in the leaf extract of *M. heterophylla* (17). The toxicity profiling of such an important plant is very helpful to appraise the safety; however, there have been no reports on the antioxidant and antimicrobial activities of the flavonoid fraction of this plant in the literature. The aim of this study was to isolate flavonoids from the leaf extract and to investigate the antioxidant and antimicrobial effects and to explore its safety in rats.

MATERIALS AND METHODS

Samples of the fresh leaves of *M. Heterophylla* were obtained from Niger State and identified by Botanist at the Department of Biological science Federal University of Technology, Minna, Nigeria. Healthy albino rats were procured from animals holding units of School of Life Sciences, Federal University of Technology, Minna, Nigeria. They were allowed unrestricted access to rat food and water. Ascorbic acid (Merck Co.; Germany), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Co.; USA). All biochemical assay kits were either obtained from Randox Laboratories Ltd, United Kingdom or Agape Diagnostics, Switzerland. All other chemicals were of analytical grade.

Isolation of Flavonoids: The leave samples were washed and dried for 2 weeks at 37°C, and ground using a grinder mill. A 50g of the plant material was extracted with 200mL of methanol, using soxhlet apparatus and the resulting extract was concentrated in a rotary evaporator. The methanol extract was dissolved in distilled water and extracted with n-butanol mixed with distilled water. The butanol extract was subjected to column chromatography on silica gel, eluted with n-hexane and methanol yielding 7.6g of a crude flavonoids mixture according to the method described by Jouad *et al*. (18).

Antibacterial Assay: The following bacteria: *Pseudomonas aeuruginisa, Salmonella typhi, Klebsiella pneumonia, Staphylococcus aureus and Escherichia coli* were the species used for the experiments. Organisms were isolated by standard methods, maintained on agar plates and refrigerated until further use. The antibacterial activity of the extract was carried out using agar-well diffusion method as described by Tsado *et al*. (19). A broth micro-dilution method (20) was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract in triplicates.

Antioxidant study: At varying concentrations (2.5-100µg/mL) of the flavonoid, using ascorbic acid as the reference drug, the radical scavenging activity was measured by2, 2′-diphenyl-1- picrylhydrazyl (DPPH) assay (21). Fe3+ ion reducing power of the flavonoid was evaluated as described by Oyaizu (22).

Toxicological Study: The acute toxicity of the flavonoid was evaluated as described by the Organization for Economic Co-operation and Development (OECD, 2001). The chronic toxicity of the flavonoid fraction was tested according to the method described by Shittu *et al*. (23). Briefly, a total of 15 rats were randomly divided into three groups of five rats each. Group 1 rats were orally given normal saline (10 mL/kg) to serve as the control. Groups 2 and 3 received 50 mg/kg and 100 mg/kg flavonoid fraction of *M. heterophylla,* respectively, for 28 days. On the 29th day, animals were denied food for 12 h and were sacrificed using diethyl ether anaesthesia. Blood samples were collected, centrifuged and the sera were prepared for biochemical analyses (24). The organs including; liver, kidney, heart, spleen and intestine were collected, washed and weighed. The relative organ weights were determined using the following formula:

Organs/body weight: The absolute organ weight (g) rat body weight on scarifies day (g)

Biochemical Parameters: The activities or concentrations of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total proteins, albumin, bilirubin, urea, potassium, creatinine, sodium, and chloride in the sera of the rats were determined by standard methods (25-29) on a spectrophotometer.

Data Analysis: Data generated were analyzed using statistical package for social science (SPSS). Differences between groups were compared by analysis of variance

(ANOVA) followed by Duncan's Multiple Range Test. The significance level was considered P<0.05.

Ethical Approval: The principles governing the use of laboratory animals as laid out by the Federal University of Technology, Minna Committee on Ethics for Medical and Scientific Research and also existing internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care Guidelines and Protocol Review were duly observed.

RESULTS

Antioxidants Activities: Flavonoid fraction of *M. heterophylla* produced progressive inhibition of DPPH radicals with increasing concentrations. The IC_{50} recorded were 33.07±0.84 µg/mL & 36.44±1.78µg/mL for the flavonoid fraction and ascorbic acid, respectively (Table 1). The ability to transform Fe^{3+} to Fe^{2+} as illustrated in Table 2 reflected the IC_{50} value of 38.08±0.89 µg/mL and 24.39±0.46 µg/mL for the flavonoid fraction and ascorbic acid, respectively.

Table 1. DPPH radical scavenging activities of flavonoid fraction of *M. Heterophylla.*

Conc. (µg/mL)	Flavonoid extract	Ascorbic Acid
5	19.35±0.45	18.56±1.64
10	29.35±0.45	42.73±3.45
20	47.43±0.56	55.89±3.21
40	69.34±0.32	69.34±4.34
80	81.56±0.25	77.94±2.34
100	93.56±0.34	85.34±4.32
IC_{50}	**33.07**±0.84	**36.44±1.78**

Values are mean ± SEM of 3 determinations.

Antimicrobial Activities: The flavonoid fraction of*M. heterophylla* produced dose-dependent inhibition of *S. aureus, E.coli, P. aeruginosa, K. pneumonia* and *S. typhi* (Table 3). *Klebsiella pneumonia* was more susceptible to the extract with the highest inhibitory concentration of 25.76±0.85 mm, followed by *E. coli* (24.98±1.89 mm), *P. aeruginosa* (23.87±0.93 mm) and *S. aureus* (21.06±0.32 mm), while the least inhibitory concentration of 20.34±1.23 mm was recorded for *S. typhi* (Table 3). The MIC of the extract was 12.5 µg/mL against all organisms tested except for *S. typhi* (25 µg/mL). The MBC were 50µg/mL against *S. aureus*, *E. coli* and 100 µg/mL against *K. pneumonia* and *S. typhi* (Table 4).

Toxicological Properties: No death was recorded in experimental animals upon administration of the extract at 5000 mg/kg during acute toxicity (LD_{50}>5000 mg/kg). A 28-day administration of flavonoid fraction of (50 and 100 mg/kg) to rats significantly (p<0.05) decrease the creatinine and bilirubin concentrations and increased AST activities, compared to those for the controls. However, total protein, albumin, ALT, ALP, Urea, chloride, potassium and sodium concentrations were similar to those of the control group (Table 5). Also, the body weight gain and organs-body weights ratios in treated groups were not significantly (p>0.05) different from those of the controls (Table 6).

Table 2. FRAP activity of leaf extracts of flavonoid Fraction of *M. Heterophylla.*

Conc. µg/mL	Flavonoid	A. Acid
100	77.45±0.94	93.95±1.05
80	65.43±0.11	84.08±1.67
40	52.35±0.32	78.40±0.533
20	45.67±0.18	60.56±0.59
10	32.13±0.94	43.03±0.75
50	21.56±0.38	29.79±0.84
2.5	9.56±0.29	18.68±0.92
IC_{50}	**38.08**±0.89	**24.39±0.46**

Values are mean ± SEM of 3 determinations.

Table 3. Susceptibility of the test organisms to the flavonoid fraction of *M. heterophylla.*

	Zone of Inhibition (mm)				
M. heterophylla	**S. aureus**	**E. coli**	**P. aeruginosa**	**K. pneumoniae**	**S.typhi**
25 µg/mL	17.87±0.30	17.98±0.94	18.23±0.22	15.97±0.98	10.34±0.34
50 µg/mL	17.90±0.53	18.05±0.77	18.96±0.45	18.95±0.83	12.67±1.05
75 µg/mL	19.89±0.98	22.54±1.98	21.08±1.09	21.56±0.98	15.83±0.86
100 µg/mL	21.06±0.32	24.98±1.89	23.87±0.93	25.76±0.85	20.34±1.23
Ampicloxbechem*	39.09±1.97	36.96±1.93	26.98±0.87	36.89±1.07	39.45±0.79

Values are Mean ± SEM of triplicate determinations. Values with the same superscript alphabets are not significantly different ($P \leq 0.05$). * = 30 µg/mL

Table 4. The minimum inhibitory (MIC) and bactericidal (MBC) concentrations of the flavonoid fraction of *M. Heterophylla.*

Test organism	MIC µg/mL	MBC µg/mL
P. aeuruginisa	12.5	50
Klebsiella pneumoniae	12.5	100
Salmonella typhi	25	100
Staphylococcus aureus	12.5	50
Escherichia coli	12.5	50

Table 5. Effects of flavonoid fraction of *M. heterophylla* on serum biochemical parameters in rats.

Biochemical Parameter	Extract (Mg/kg bw)		
	50	100	Control
Protein (mg/dl)	37.89±2.96[a]	38.82±4.03[a]	37.57±2.78[a]
Bilirubin (mg/dl)	3.02±0.32[a]	2.71±0.22[a]	5.32±0.46[b]
Albumin (mg/dl)	3.04±0.78[a]	3.26±0.56[a]	3.43±0.36[a]
ALT(U/L)	4.98±1.45[a]	5.44±2.34[a]	5.45±0.24[a]
AST(U/L)	43.45±4.32[b]	34.56±4.32[ab]	28.30±2.35[a]
ALP (U/L)	138.97±12.38[a]	132.41±7.13[a]	141.35±3.56[a]
Creatinine	8.45±1.34[a]	10.73±0.96[ab]	11.34±0.45[b]
Urea	27.05±3.56[a]	29.63±3.75[a]	26.78±2.46[a]
Chloride	216.78±3.45[a]	234.56±3.57[a]	222.57±4.56[a]
Potassium	6.23±0.29[a]	6.32±0.39[a]	6.20±0.88[a]
Sodium	24.45±3.45[a]	25.08±2.34[a]	22.67±2.45[a]

Values are mean ± SEM of 5 determinations. Values along the same row with different superscripts are significantly different ($p<0.05$).

Table 6. Relative organ weight ratio of rats administered flavonoid fraction of *M. Heterophylla.*

ROUPS	Liver	Heart	Intestine	Lungs	Kidney	Spleen
50	2.34±0.19[a]	0.89±0.00[a]	3.56±0.03[a]	1.56±0.00[a]	0.87±0.02[a]	0.44±0.00[a]
100	2.05±0.09[a]	0.77±0.00[a]	3.06±0.03[a]	1.23±0.00[a]	0.89±0.00[a]	0.42±0.05[a]
Control	2.38±0.56[a]	0.84±0.00[a]	3.45±0.01[a]	1.59±0.00[a]	0.79±0.00[a]	0.45±0.01[a]

Values are mean ± SEM of 5 determinations. Values along the same column with different superscripts are significantly different ($p<0.05$).

DISCUSSION

Generally, natural products particularly from plant extracts have been reported for their pharmacological properties (5). It was reported that crude extract of *M. heterophylla* exhibited significant antioxidant and antimicrobial properties (16). Moreover, previous phytochemical studies on *M. Heterophylla* revealed the presence of different classes of secondary metabolites particularly phenols and flavonoids, which have been implicated in the pharmacological activities of the plant extract (30, 31).

The results of antioxidant activity showed that the flavonoid fraction of *M. heterophylla* had DPPH and FRAP radical scavenging activities in a dose–dependent manner (Tables 1 and 2). Our results are in line with that of a previous study (19) that reported that the antioxidant potentials of *Newbouldia laevis* and *Crateva adansonii* leaf extracts increased as the concentration increased. The activities recorded for the flavonoid (IC_{50} =33.07±0.84 µg/mL) was higher than that of ascorbic acid (33.07±0.84 µg/mL). Also, the activities of the flavonoid were significantly better than the scavenging properties reported for some medicinal plants, such as *Padina pavonica* (IC_{50} = 5.59 mg/ml), *Laurenica majuscule* (IC_{50} =14.3 mg/ml), and *Laurencia catarinensis* (IC_{50} = 53.8 mg/ml) (32). These potent scavenging activities could be very useful in the management of certain neurodegenerative disorders, AIDS and cancers (5,6).

Antimicrobial activity is one of the important properties of flavonoid compounds. The results of the MICs revealed that both the gram positive and gram-negative bacteria tested were susceptible to the flavonoid fraction of *M. heterophylla*. The MIC values ranged from 12.5µg/mL to 25µg/mL for both gram-positive and gram-negative bacteria except for *S. typhi* (25µg/mL). The inhibition of bacterial strains (*S. Aureus* & *E. coli*) suggests that the flavonoid possesses broad spectrum antibacterial properties, which could be used in the treatment of skin diseases and food poisoning, in which these pathogens are often implicated (19). They inhibit the hydrolytic enzymes (proteases), microbial adhesion, and cell envelope transport proteins (6). In addition, flavonoids form complexes with soluble and extra cellular proteins of bacterial cell walls leading to their death (33).

The need for the analysis of biochemical parameters following subacute administration of plant extracts has been suggested by previous studies (23, 24,34), which have revealed that some alterations in serum concentrations of biochemical parameters, such as AST, ALT, ALP, total proteins and bilirubin are the indicators of hepatocellular damage, compromised cell membrane integrity, hepatitis, cirrhosis, and bile duct obstruction (35). Similarly, the concentration of serum electrolytes, urea, and creatinine reflect the secretory and excretory roles of kidney (34). Consequently, alterations in the serum concentrations of creatinine, bilirubin and AST observed in this study suggest that the normal function of the rats' kidneys and liver had been hampered (23). Such alteration could, negatively affect the metabolic activities of the liver and consequently the health of the animals. The significant alterations in the serum creatinine concentrations could reflect the flavonoid ability to interfere with its metabolism (24).

Interestingly, the 28-day administration of flavonoid fraction to rats did not cause any significant alterations to the levels of serum total protein, albumin, ALT, ALP, Urea, chloride, potassium and sodium compared with the control values. This simply implies that the functional integrity of liver and kidney cells had not been compromised. Similarly, since ALT is more specific to liver than AST, the insignificant decrease in serum ALT seen in flavonoid treated groups suggests that the extract does not have hepatotoxic effects (23). Moreover, it is worth mentioning that this is the first

study that investigated the antioxidants, antimicrobial and safety of the flavonoid fraction of *M. heterophylla*

CONCLUSIONS

The Flavonoid fraction of *M. heterophylla* is relatively non-toxic on acute and chronic exposures at 50-100 mg/kg of the experimental rats with the potential to serve as a candidate for the development of antimicrobial and antioxidant drug.

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CONFLICT OF INTEREST

The authors declare no conflict of interest existed while conducting this study.

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